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**“Novel Approach to Study Virus – Host Interactions in the
Prototypic Murine Infection Model of Lymphocytic
Choriomeningitis Virus”**

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Inhaltsverzeichnis

Seite

4	Summary / Zusammenfassung
5-10	“Recovery of an arenavirus entirely from RNA polymerase I/II-driven cDNA” (<i>akzeptiert zur Veröffentlichung in den Proceedings of the National Academy of Sciences, 2006</i>)
11-12	Bestätigung der Redaktion über die Annahme des Manuskripts
13	Lebenslauf
14	Danksagungen

Summary

The prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) has represented a primary workhorse of viral immunologists for almost a century, and it has served as an important model to study basic principles of the arenavirus molecular biology. Its negative-stranded bisegmented RNA genome had however posed a major obstacle to attempts at manipulating the infectious virus by reverse genetic techniques.

Here we report the recovery of infectious LCMV (strain Armstrong clone 13) entirely from cDNA. Intracellular transcription of the short and the long viral genome segment from polymerase I-driven vectors and coexpression of the minimal viral trans-acting factors NP and L from polymerase II-driven plasmids resulted in the efficient formation of infectious virus with genetic tags in the viral genome segments. The cDNA-derived viruses behaved identical to wild type virus, both in cell culture and in infected mice. Importantly, they caused a chronic infection and suppressed the adaptive immune response to an unrelated third party virus. This technology provides an important basis to investigate viral determinants of persistent infection and immunosuppression. In addition, our findings demonstrate that polymerase I-/II-based vector systems may represent an efficient alternative strategy for the recovery of cytoplasmic negative-strand RNA viruses from cDNA.

Zusammenfassung

Das Arenavirus Lymphozytäre-Choriomeningitis-Virus (LCMV) war eines der Standardmodelle für Virusimmunologen in den letzten Jahrzehnten. Sein zweisegmentiges negativsträngiges RNA Genom hat dabei aber ein großes Hindernis dargestellt für die Bemühungen, das Virusgenom mittels reverser Genetik zu manipulieren.

Hier zeigen wir die Generierung von infektiösem LCMV (Stamm Armstrong Clone 13) komplett aus cDNA. Intrazelluläre Transkription des langen und des kurzen Segments durch Polymerase-I Vektoren sowie Koexpression der minimal-notwendigen viralen *trans-acting* Elemente NP und L von Polymerase-II Plasmiden führte zu der effizienten Bildung von infektiösem Virus mit genetischen Markierungen in seinen beiden Genomsegmenten. Die aus cDNA generierten Viren verhielten sich ident zum Wildtypvirus sowohl in Zellkultur als auch in infizierten Mäusen. Dabei führten sie –wie das Wildtypvirus– zu einer chronischen Infektion und supprimierten die adaptive Immunantwort gegenüber einem nichtverwandten Virus. Diese Technologie liefert die Basis für die Erforschung von viralen Determinanten für persistente Infektionen und Immunsuppression. Zusätzlich demonstrieren unsere Ergebnisse, daß Polymerase-I-/II basierende Vektorsysteme eine effiziente alternative Strategie für die Generierung von zytoplasmatischen negativsträngigen RNA Viren aus cDNA darstellen.

Recovery of an arenavirus entirely from RNA polymerase I/II-driven cDNA

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The prototypic arenavirus lymphocytic choriomeningitis virus has been a primary workhorse of viral immunologists for almost a century, and it has served as an important model for studying basic principles of arenavirus molecular biology. Its negative-stranded bisegmented RNA genome has, however, posed a major obstacle to attempts at manipulating the infectious virus by reverse genetic techniques. Here, we report the recovery of infectious lymphocytic choriomeningitis virus (the immunosuppressive strain clone 13) entirely from cDNA. Intracellular transcription of the short and the long viral genome segment from polymerase (pol) I-driven vectors and coexpression of the minimal viral-transacting factors NP and L from pol II-driven plasmids resulted in the efficient formation of infectious virus with genetic tags in both genome segments. The cDNA-derived viruses behaved identically to wild-type virus in both cell culture and infected mice. Importantly, they caused a chronic infection and suppressed the adaptive immune response to an unrelated third-party virus. This technology provides an important basis for investigating viral determinants of persistent infection and immunosuppression. In addition, our findings demonstrate that pol I/II-based vector systems may represent an efficient alternative strategy for the recovery of cytoplasmic negative-strand RNA viruses from cDNA.

immunosuppression | lymphocytic choriomeningitis virus | reverse genetics | negative-strand RNA virus

The prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) was first isolated in the 1930s (1, 2). Ever since, it has served as a primary workhorse in the fields of immunology and viral pathogenesis, contributing substantially to the development of multiple key concepts in both disciplines (reviewed in ref. 3). Among its many interesting aspects, LCMV has been particularly instrumental in the investigation of antiviral tolerance and virus-induced immunosuppression in persistent infection. Moreover, this virus may represent a neglected human pathogen (4). Over the years it has been found that, in addition to LCMV, a substantial number of other arenaviruses are widely distributed in the rodent populations of the entire world. Some of them, e.g., Lassa fever virus, a close relative of LCMV, and the New World viruses Junin, Machupo, and Guanarito, are of particular interest because they can cause hemorrhagic fevers with high lethality when accidentally transmitted to humans (5).

Arenavirus genomes consist of two single-stranded RNA segments, each of them encoding for two viral genes in ambisense orientation, separated by an intergenic region (IGR) that is predicted to fold into a secondary RNA structure of high stability. The short segment (S segment) expresses the viral surface glycoprotein (GP) and the nucleoprotein (NP) that encapsidates the viral genome and replicative intermediates (Fig. 1A). The long segment (L segment) carries the genes for the viral RNA-dependent RNA polymerase (pol) L and the small matrix protein Z. Despite their ambisense coding strategy, however, arenaviruses are negative-strand viruses, i.e., transfection of purified viral RNA into permissive cells does not initiate an infectious cycle, a fact that has long impeded the analysis of arenavirus molecular biology. Only relatively recently, a mini-

replicon system has been developed, allowing for the analysis of the viral cis-acting elements and transacting factors involved in transcription, replication, and particle formation (6–10), and similar systems have subsequently been described for Tacaribe virus and Lassa fever virus (11, 12). The LCMV reverse genetic system had initially been based on the intracellular expression of a viral model genome (MG) from T7 RNA pol (T7), combined with either T7-driven or pol II-driven coexpression of the minimal viral-transacting factors NP and L (6, 7). Owing to the virus's cytoplasmic life cycle, T7 had been the obvious choice for expression of an LCMV genome analogue. For orthomyxoviruses with a nuclear life cycle, however, pol I-driven vectors had long been accepted as a powerful method for expressing MGs and later for the efficient recovery of infectious viruses entirely from plasmid (reviewed in ref. 13). Stimulated by findings made in the bunyavirus field (14), we have also documented that pol I-driven vectors represented an efficient alternative means for expressing LCMV MG RNAs (9). Pol I-derived MG RNA species were exported to the cytoplasm where they assembled with plasmid-supplied L and NP proteins into ribonucleoproteins (RNPs) that were active in cytoplasmic transcription and replication (9). This system could even be exploited to reconstitute a recombinant full-length S-segment RNP. Upon reassortment with helper virus as a source for the L segment, an infectious LCM virus with an engineered S segment was recovered that expressed the GP of vesicular stomatitis virus (VSV) instead of the LCMV-GP (rLCMV/VSVG, ref. 15). In a reverse reassortment process, the VSVG-expressing S segment of this virus could be exchanged for a cDNA-derived S segment of interest, a strategy that was vastly facilitated by the potent selective capacity of VSV-neutralizing antibodies. Accordingly, viruses generated by this method have allowed us to study the role of the viral GP in liver disease (A.B., D. Merkler, E. Horvath, L. Bestmann, and D.D.P., unpublished work) and GP-based strategies to attenuate arenaviruses for use as live vaccines (A.B., N. U. Gerber, D. Merkler, E. Horvath, J.C.d.l.T., and D.D.P., unpublished work). Yet the arenavirus L segment harbors key determinants of the viral phenotype (16) that could not be studied in a reverse genetic manner. Here, we report that the intracellular expression of the LCMV RNA genome from pol I-driven vectors, combined with pol II-driven coexpression of the minimal transacting factors NP and L, resulted in the efficient recovery of infectious LCMV entirely from cDNA. This finding shows that pol I-driven systems may represent an efficient

Conflict of interest statement: No conflicts declared.

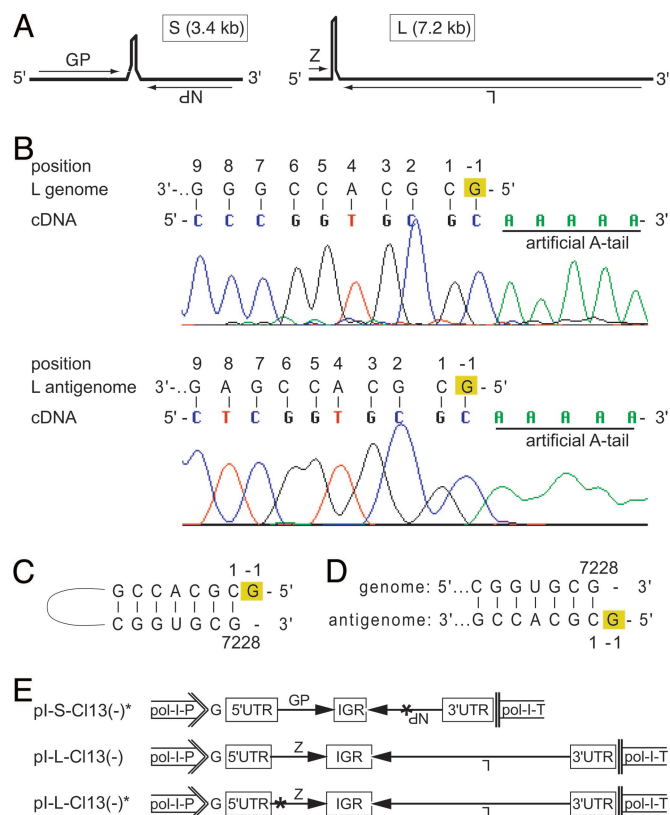
Abbreviations: LCMV, lymphocytic choriomeningitis virus; S segment, short segment; L segment, long segment; GP, glycoprotein; NP, nucleoprotein; MG, model genome; pol, polymerase; VSV, vesicular stomatitis virus; C13, clone 13; wtC13, wild-type C13; ARM, Armstrong; IGR, intergenic region; L-IGR, L segment-IGR; RT, reverse transcription; PFU, plaque-forming units.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. DQ361065 (S segment) and DQ361066 (L segment)].

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alternative for the recovery of cytoplasmic negative-strand RNA viruses from cDNA. Moreover, the technology presented here and a simultaneously developed T7-driven system for the recovery of LCMV from cDNA (34) will provide powerful reverse genetic approaches for the study of the arenavirus immunobiology by using genetically engineered replication-competent viruses.

Results

We had observed that an LCMV L segment-based MG of ≈ 7.6 kb could be expressed from a pol I-driven vector and yielded reporter gene activity when the viral-transacting factors were coexpressed (D.D.P. and J.C.d.I.T., unpublished work). This finding lent support to the hypothesis that pol I should be able to express not only a full-length S segment (15) but also the L segment of LCMV (7.2 kb in length) for recognition by the viral-transacting factors. Thus, we set out to extend our previously published pol I/II-driven reverse genetic system for LCMV (9, 10, 15) to allow for the recovery of infectious virus entirely from plasmid. The previously published MG system and a partial

rescue system for LCMV had been based on the Armstrong (ARM)53b strain (6, 9) that is unable to establish persistence in adult mice (17–19). With the intention of studying the virus–host relationship during chronic infection we therefore attempted to establish a complete rescue system for the ARM-derived variant clone 13 [Cl13 (17)] rather than for ARM itself. We first revisited the published Cl13 sequence [GenBank accession no. DQ361065 (S segment) and GenBank accession no. DQ361066 (L segment), compare with GenBank accession nos. M64450, J04331, and M27693, and ref. 20; for technical details see *Supporting Text*, which is published as supporting information on the PNAS web site] with an emphasis on the noncoding sequences of the L segment. The L-segment IGR (L-IGR) had previously been reported to exhibit substantial variability in length (21). Considering the important roles of the S-segment IGR in the viral life cycle (10), we reasoned that a correct L-IGR sequence was likely of importance for the efficient recovery of LCMV from plasmid. To overcome the intrinsic difficulties related to the predicted secondary RNA structures in the L-IGR, we used a heat-stable reverse transcription (RT) pol with helicase activity, followed by PCR amplification (for details see *Supporting Text*). Sequence analysis of the obtained PCR product readily yielded an L-IGR consensus sequence that was of the same length, although not of identical sequence, as the one recently published for LCMV-ARM53b (GenBank accession no. DQ361066, compare with GenBank accession nos. AY847351 and AY894816).

It had been demonstrated that arenavirus S segments carry a nontemplated G residue at their 5' ends (22), but the precise 5' terminus of the LCMV L segment was less certain (21, 23). For a Lassa fever virus S segment analogue it had, however, been shown that efficient template recognition by the viral RNA-dependent RNA pol depended critically on the precise 5' end (12), and we have made similar observations for the LCMV S segment (ref. 8 and D.D.P. and J.C.d.I.T. unpublished work). Thus, we analyzed the CI13 L segment 5' ends by using a 5' RACE protocol that was based on A-tailing of first-strand cDNA with terminal deoxynucleotidyl transferase (for technical details see *Supporting Text*). This approach unambiguously revealed a G residue at position -1 on both the LCMV L-segment genome and antigenome (Fig. 1B). Different investigators (24-26) have unequivocally shown that the 3' end of the L-segment genome terminates on a G residue and not on a C (Fig. 1C and D). Thus, intramolecular annealing of the genome into a panhandle structure [the putative viral promoter (8), Fig. 1C] does not form a flush end as for most negative-strand RNA viruses (22). Also, this finding suggests that the 5' G of antigenomes found here represents a nontemplated base like in the S segment (ref. 22 and Fig. 1D). An analogous conclusion for the L-segment genome 5' G, although likely, however, awaits formal determination of its template, i.e., of the antigenome 3' end.

Based on this sequence information we assembled Cl13 S- and L-segment cDNAs containing the master consensus sequence and cloned them in genomic polarity under control of the mouse pol I promoter and terminator cassettes (14). Thereby we obtained the plasmids pI-S-Cl13(-)* and pI-L-Cl13(-), respectively (Fig. 1E). pI-S-Cl13(-)* was partially based on a previously published backbone [pS-Bsm(-) (15)] that contained a noncoding single-nucleotide tag, deleting an EcoNI site for discrimination from wild-type virus (Fig. 2C). In contrast, pI-L-Cl13(-) was assembled *de novo* and did not contain a genetic tag. A second constructs was also generated [pI-L-Cl13(-)*, Fig. 1E] that differed from the wild-type Cl13 (wtCl13) sequence by a noncoding single-nucleotide transition in the Z ORF. Thereby an additional AvaII recognition site was introduced (Fig. 2C). The NP ORFs of ARM53b and Cl13 are identical, and hence the available pol II-driven expression plasmid pC-NP (7) could also be used here for intracellular coexpression of this viral protein. The previously published plasmid for intracellular ex-

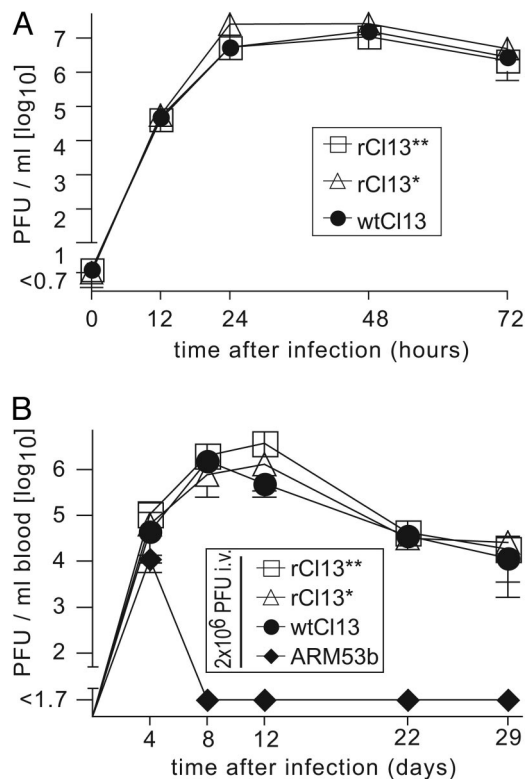


Fig. 3. Propagation of cDNA-derived viruses in cell culture and infected mice. (A) Subconfluent BHK-21 cells in M6 tissue culture wells were infected with wtCl13, rCl13*, or rCl13** at multiplicity of infection of 0.01. Supernatant was collected at the indicated time points for determination of viral titers. Symbols represent the mean \pm SD of three individual culture wells. (B) C57BL/6 mice were infected with 2×10^6 PFU of ARM53b, wtCl13, rCl13*, or rCl13**. Blood was collected at the indicated time points for the assessment of viral titers. Symbols represent the mean \pm SD of four mice per group. One representative experiment of two is shown.

day 29 that were in a similar range as in mice that had not undergone prior infection with LCMV (Fig. 4A). In marked contrast, these responses were suppressed to below detection levels in mice that were persistently infected with wtCl13, rCl13*, or rCl13**. The early IgM response (i.e., day 3 after VSV infection) is known to be T cell-independent, whereas isotype class switch and the production of IgG is CD4⁺ T cell-dependent, indicating that the cDNA-derived viruses rCl13* and rCl13** shared the ability of wtCl13 to suppress both of these immune functions. Moreover, persistent infection with one of these three viruses caused a marked suppression of the VSV-specific CD8⁺ T cell response, whereas ARM infection did not (Fig. 4B). As expected, persistent infection with wtCl13, rCl13*, or rCl13** also resulted in the exhaustion of the CD8⁺ T cell response to the immunodominant LCMV epitope NP396, whereas NP396-specific IFN- γ -producing CD8⁺ T cells were readily detected in ARM-infected mice (Fig. 4B). Taken together, the *in vivo* behavior of the cDNA-derived and genetically tagged rCl13* and rCl13** viruses was indistinguishable from wtCl13, validating our pol I-based reverse genetic system for the analysis of the virus–host relationship in adult persistent LCMV infection. Importantly, two viruses recovered from different L-segment expression plasmids (rCl13* and rCl13**) exhibited an identical phenotype. This is an additional indication for the high reproducibility of our reverse genetic system, a property that will be of paramount importance for future studies addressing the impact of individual point mutations on viral immunobiology.

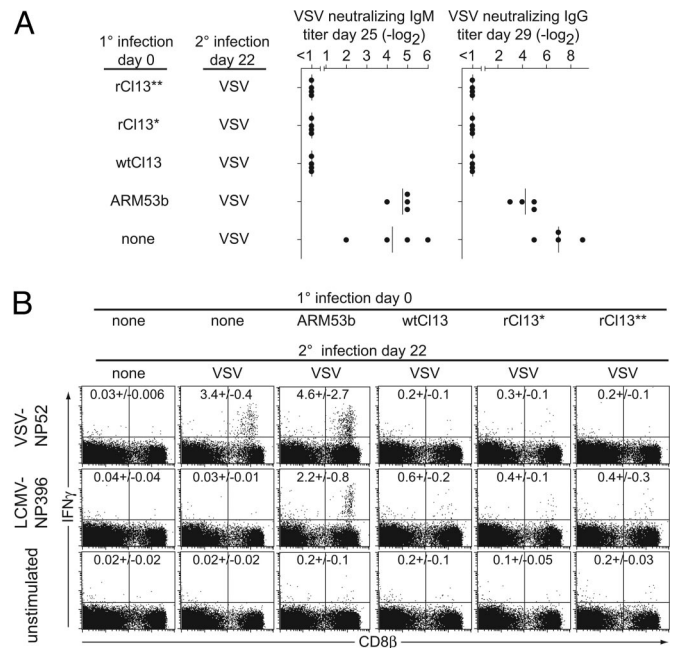


Fig. 4. Suppression of B cell and T cell responses by the cDNA-derived viruses. On day 0, C57BL/6 mice (same mice as in Fig. 3B) were infected i.v. (1° infection) with 2×10^6 PFU of ARM53b, wtCl13, rCl13*, or rCl13**, or they were left uninfected (none). Twenty-two days later, the indicated groups of mice were challenged with 2×10^6 PFU of VSV i.p. (2° infection). (A) On days 25 and 29 (3 and 7 days after secondary infection), serum samples were collected for determination of VSV-neutralizing IgM (day 25) and neutralizing IgG (day 29). Symbols represent individual mice. One representative experiment of two is shown. (B) On day 30, the mice were killed, and the frequency of splenic IFN- γ -producing CD8⁺ T cells specific for either the immunodominant VSV NP-derived epitope NP52 or the LCMV NP-derived epitope NP396 was determined in an intracellular cytokine assay. Background IFN- γ production is shown for comparison (unstimulated). The frequency of IFN- γ -producing CD8⁺ cells within the total CD8⁺ T cell compartment is indicated for each representative FACS plot. Numbers indicate the mean \pm SD of three mice per group.

Discussion

Unlike for all of the other known families of negative-strand RNA viruses (13, 29), arenaviruses have to our knowledge not previously been recovered entirely from cloned cDNA, and the same seems to be the case for bona fide ambisense viruses. With this study and a simultaneous study by Sanchez and de la Torre (34), these hurdles have been overcome. Here, we have exploited pol I-driven vectors for expression of the viral genome segments, a strategy that is against current practice for a cytoplasmic virus like LCMV. A T7-based rescue system, as exploited by A. B. Sanchez and J.C.d.l.T. (34), would have represented the obvious method of choice. It has been known for a while, however, that viruses with a nuclear life cycle such as influenza A can be recovered by using either T7- or pol I-driven systems (13). As shown here, the same holds true for cytoplasmic viruses. This possibility had previously been suggested by pol I-based MG systems for bunyaviruses, filoviruses, and arenaviruses (9, 14, 30), but a complete pol I-based rescue of an infectious negative-strand virus with a cytoplasmic life cycle has to our knowledge not previously been reported. The rescue system of choice for a particular virus may therefore not depend only on its intracellular compartment of replication. The differential proneness of T7 or pol I to terminate prematurely at terminator-like sequences within a given viral genome may represent only one of many factors that may be difficult to predict but could substantially influence the efficiency of virus recovery. In addition, pol I-based systems render the use of a 3' terminal ribozyme unnecessary

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“Nothing in life is to be feared. It is only to be understood.”
Marie Curie